Electronic Supplementary Information

Unprecedented Formation of Sterically Stabilized Phospholipid Liposomes of Cuboidal Morphology

Pavel Bakardzhiev,[a] Aleksander Forys,[b] Barbara Trzebicka,[b] Tonya Andreeva,[c] Stanislav Rangelov*[a]

[a] *Institute of Polymers, Bulgarian Academy of Sciences, Akad. G. Bonchev St., Bl. 103-A, 1113 Sofia, Bulgaria*

[b] *Center of Polymer and Carbon Materials, Polish Academy of Sciences, ul. M. Curie-Skłodowskiej 34, 41-819 Zabrze, Poland*

[c] *Faculty of Applied Chemistry, Reutlingen University, Alteburgstraße 150, D-72762 Reutlingen, Germany*

Corresponding Author

* Stanislav Rangelov, Institute of Polymers, Bulgarian Academy of Sciences, Akad. G. Bonchev St., Bl. 103-A, 1113 Sofia, Bulgaria. Email: rangelov@polymer.bas.bg

- **Synthesis of DDP-PG polymers**

The synthetic route for the preparation of DDP-PG polymers is presented in Figure S1. The lipid-mimetic anchor (1,3-didodecyloxy-propane-2-ol, DDP) was prepared by a reaction of 1-dodecanol with dodecyl glycidyl ether in the presence of catalytic amounts of $SnCl_4$ as described elsewhere.[1] DDP was partially deprotonated by reacting with KOH and removal of the released water. The DDP-PEEGE precursors were obtained by anionic ring-opening polymerization of ethoxyethyl glycidyl ether (EEGE) using the partially deprotonated DDP as an initiator. In the last step, the protective ethoxyethyl groups were cleaved thus yielding DDP-polyglycidol conjugates. DDP-PG polymers were obtained by following the synthetic procedure firstly described by Bakardzhiev et al.[2]

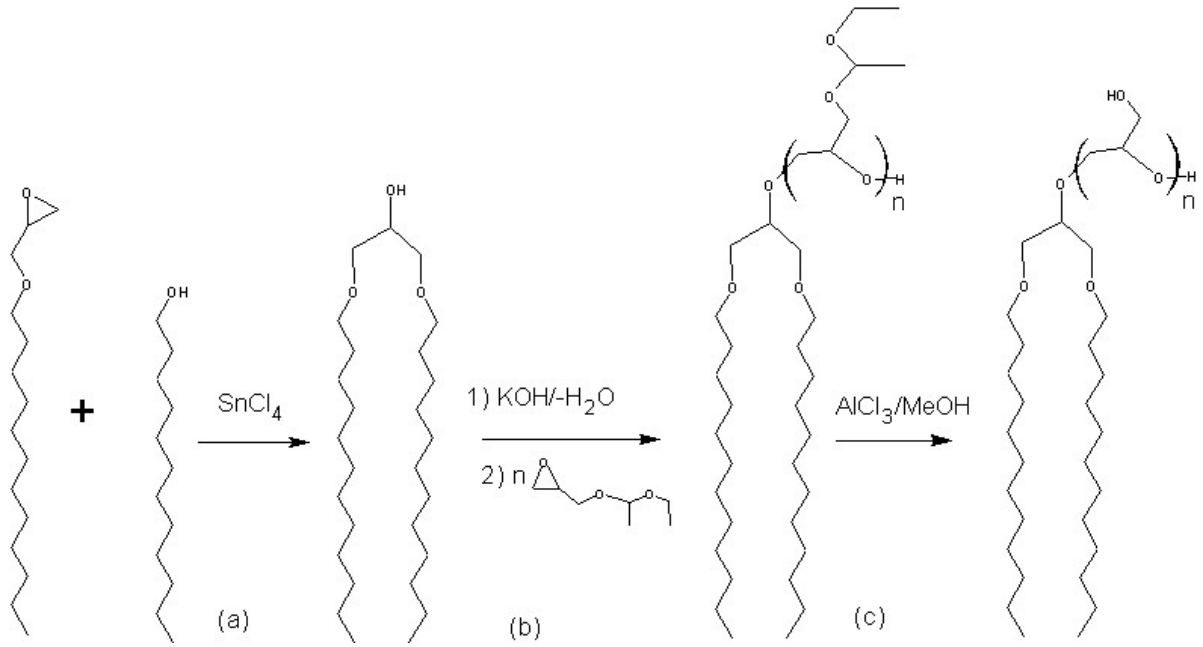


Figure S1. Synthetic pathway for the preparation of DDP-PG. n = 25, 72, 110.

- **Preparation of liposomes**

1,2-Dipalmitoyl-sn-glycero-phosphocholine (DPPC), cholesterol, chloroform and methanol were purchased from Sigma Aldrich. Chloroform solutions of DPPC and cholesterol (2:1 M ratio, 3 mM total lipid concentration) were placed into glass tubes to which a methanol solution of the respective polymer in a defined polymer/lipid molar ratio was added. The solvents were evaporated under a stream of argon and all traces of solvent were removed under vacuum

overnight. Saline solution (0.9 % NaCl in water) was added to the dry lipid/polymer film and the resulting dispersions were subjected to ten freeze–thaw cycles and then extruded 30 times through polycarbonate filters of pore size 100 nm using a LiposoFast handle type extruder (Avestin Inc., Canada). The dispersions appeared as moderately opalescent, transparent liquids (Figure S2).

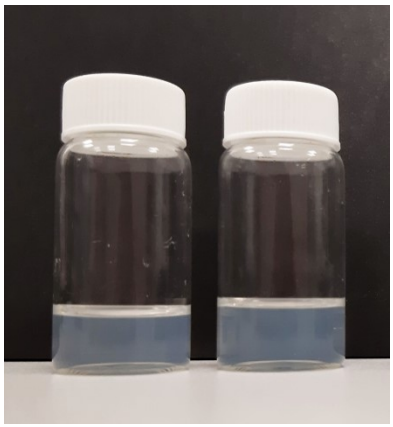


Figure S2. Vials containing liposomal dispersions of DPPC/Cholesterol stabilized by 20.0 mol % of DDP-PG72 (left) and DDP-PG110 (right).

- **Cryogenic Transmission Electron Microscopy (Cryo-TEM)**

Cryo-TEM images were obtained using a Tecnai F20 X TWIN microscope (FEI Company, Hillsboro, Oregon, USA) equipped with field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Gatan Rio 16 CMOS 4k camera (Gatan Inc., Pleasanton, California, USA) and processed with Gatan Microscopy Suite (GMS) software (Gatan Inc., Pleasanton, California, USA). Specimen preparation was done by vitrification of the aqueous solutions on grids with holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Großlöbichau, Germany). Prior to use, the grids were activated for 15 seconds in oxygen plasma using a Femto plasma cleaner (Diener Electronic, Ebhausen, Germany). Cryo-samples were prepared by applying a droplet (3 μL) of the suspension to the grid, blotting with filter paper and immediate freezing in liquid ethane using a fully automated blotting device Vitrobot Mark IV (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a cryo-TEM-holder Gatan 626 (Gatan Inc., Pleasanton, USA) and analyzed in the TEM at -178°C.

- **Dynamic light scattering (DLS)**

DLS measurements were performed on a Brookhaven BI-200 goniometer with vertically polarized incident light at a wavelength $\lambda = 633$ nm supplied by a He–Ne laser operating at 35 mW and equipped with a Brookhaven BI-9000 AT digital autocorrelator. Measurements were

made at angles θ in the 50 – 130° range. The autocorrelation functions were analyzed using the constrained regularized algorithm CONTIN[3] to obtain the distributions of the relaxation rates (Γ). The latter provided distributions of the apparent diffusion coefficient ($D = \Gamma/q^2$) where q is the magnitude of the scattering vector given by $q=(4\pi n/\lambda)\sin(\theta/2)$, n is the refractive index of the medium. The mean hydrodynamic radius was obtained by the Stokes–Einstein equation (equation 1):

$$R_h = kT/(6\pi\eta D) \qquad \text{(equation 1)}$$

where k is the Boltzman constant, η is the solvent viscosity at temperature T in Kelvin and D is the diffusion coefficient. The diffusion coefficients were determined from the slopes of the linear fit of the data plotted as relaxation rate versus $\sin^2(\theta/2)$. All measurements were performed at 25 °C at a single solute concentration.

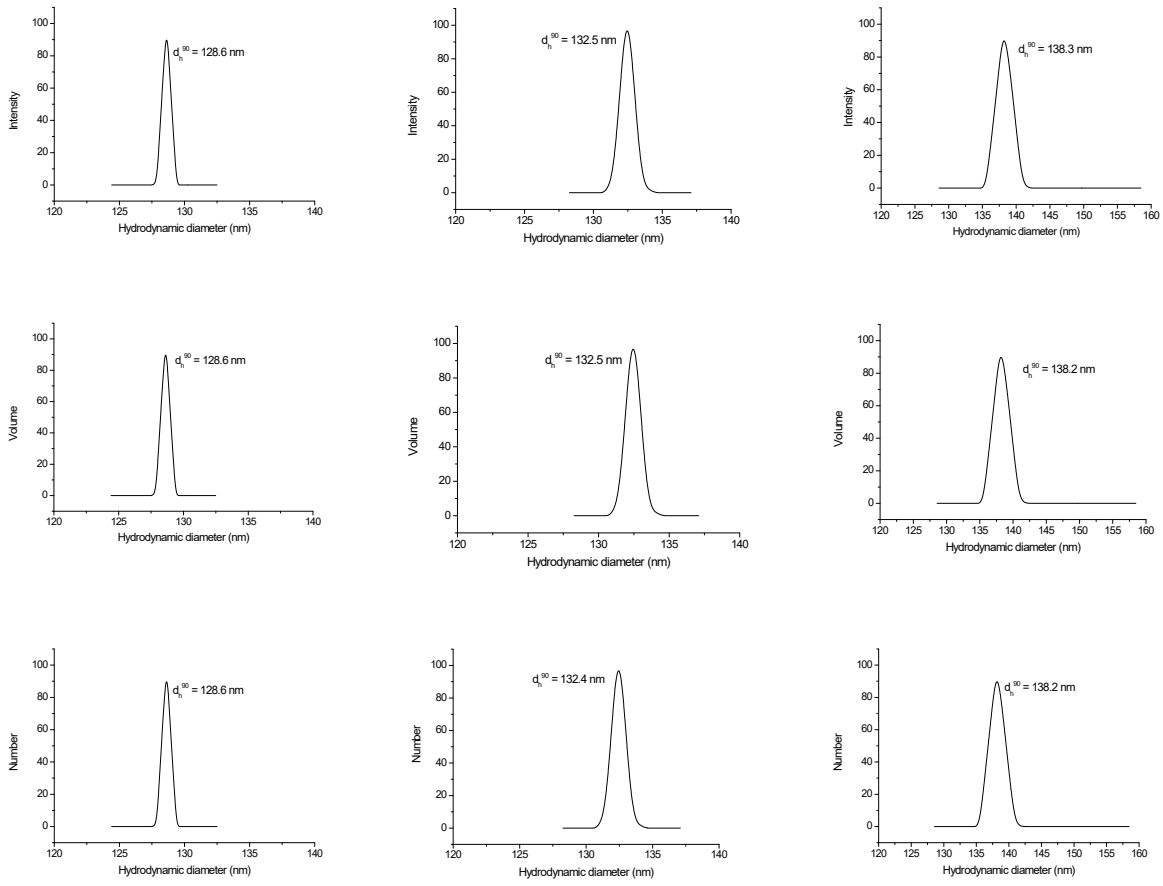


Figure S3. Particle size distribution from DLS determined by intensity (top row), volume (middle row), and number (bottom row) of vesicles DPPC/Cholesterol/DDP-PG25-12.5 (left column), DPPC/Cholesterol/DDP-PG72-12.5 (middle column), and DPPC/Cholesterol/DDP-PG110-12.5 (right column). The first and second digits indicate the polyglycidol degree of polymerization and DDP-PG content in mol %, respectively.

In Figure S3, representative particle size distributions weighted by intensity, by volume, and by number are displayed. The narrow and monomodal size distribution is further evidenced by the practically identical values of the hydrodynamic diameters, d_h, determined by intensity-, volume-, and number-weighting.

The angular dependences of the relaxation rate, Γ vs. $\sin^2(\theta/2)$, for all samples, from which the apparent diffusion coefficients were determined are shown in Figure S4.

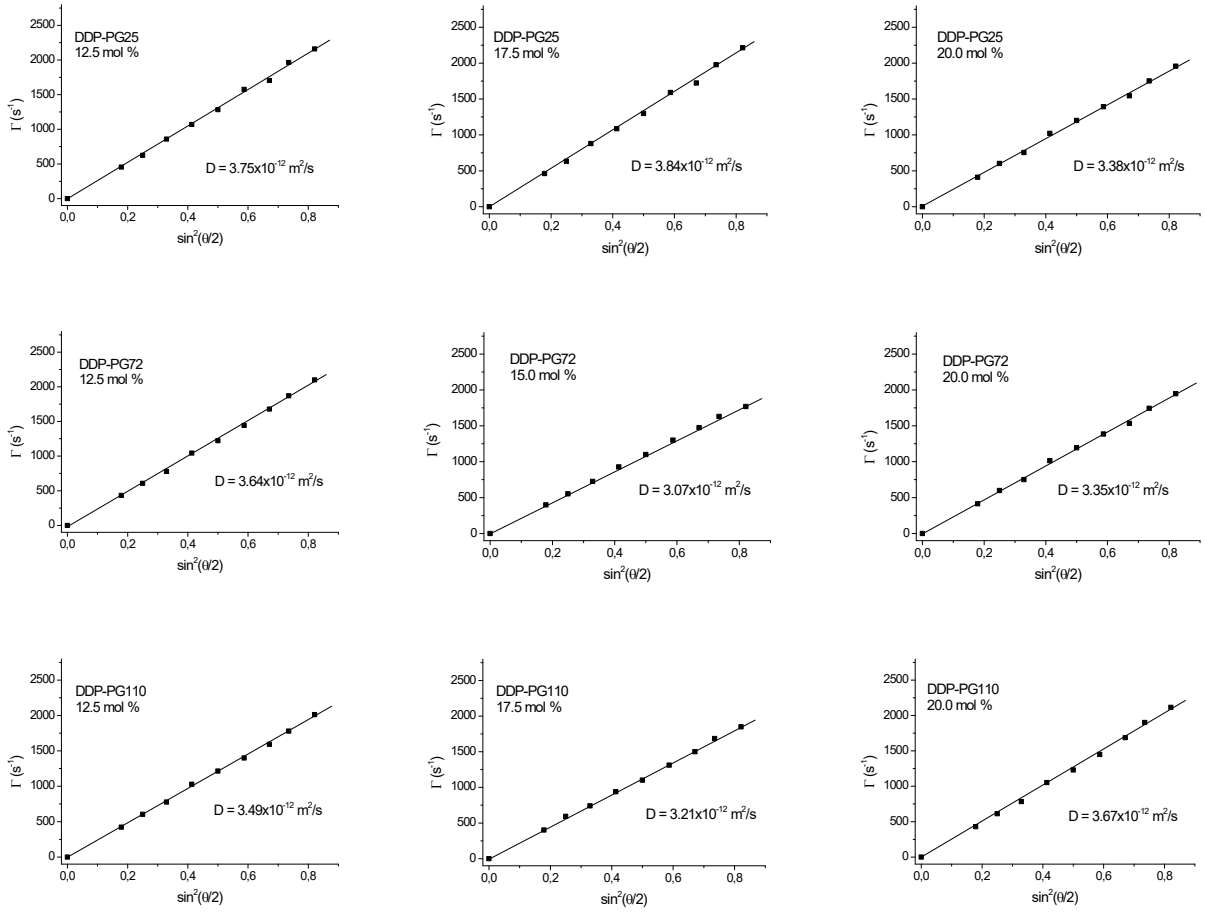


Figure S4. Angular dependence of the relaxation rate, Γ, of vesicles DPPC/Cholesterol/DDP-PG25 (top row), DPPC/Cholesterol/DDP-PG72 (middle row), and DPPC/Cholesterol/DDP-PG110 (bottom row) at different DDP-PG contents indicated.

- **Static light scattering (SLS)**

The SLS measurements were carried out in the interval of angles from 40 to 140° using a Brookhaven BI-200 goniometer with vertically polarized incident light at a wavelength λ = 633 nm supplied by a He–Ne laser operating at 35 mW. The radii of gyration, R_g, were obtained by partial Berry plots from the dependences of $I'^{-1/2}$ on q^2, where I′ is the quantity $I_{ex}\sin\theta$, with I_{ex}

being the excess of scattered light intensity, and q^2 is the scattering vector defined above. All measurements were performed at 25 °C at a single solute concentration. Partial Berry plots are shown in Figure S5.

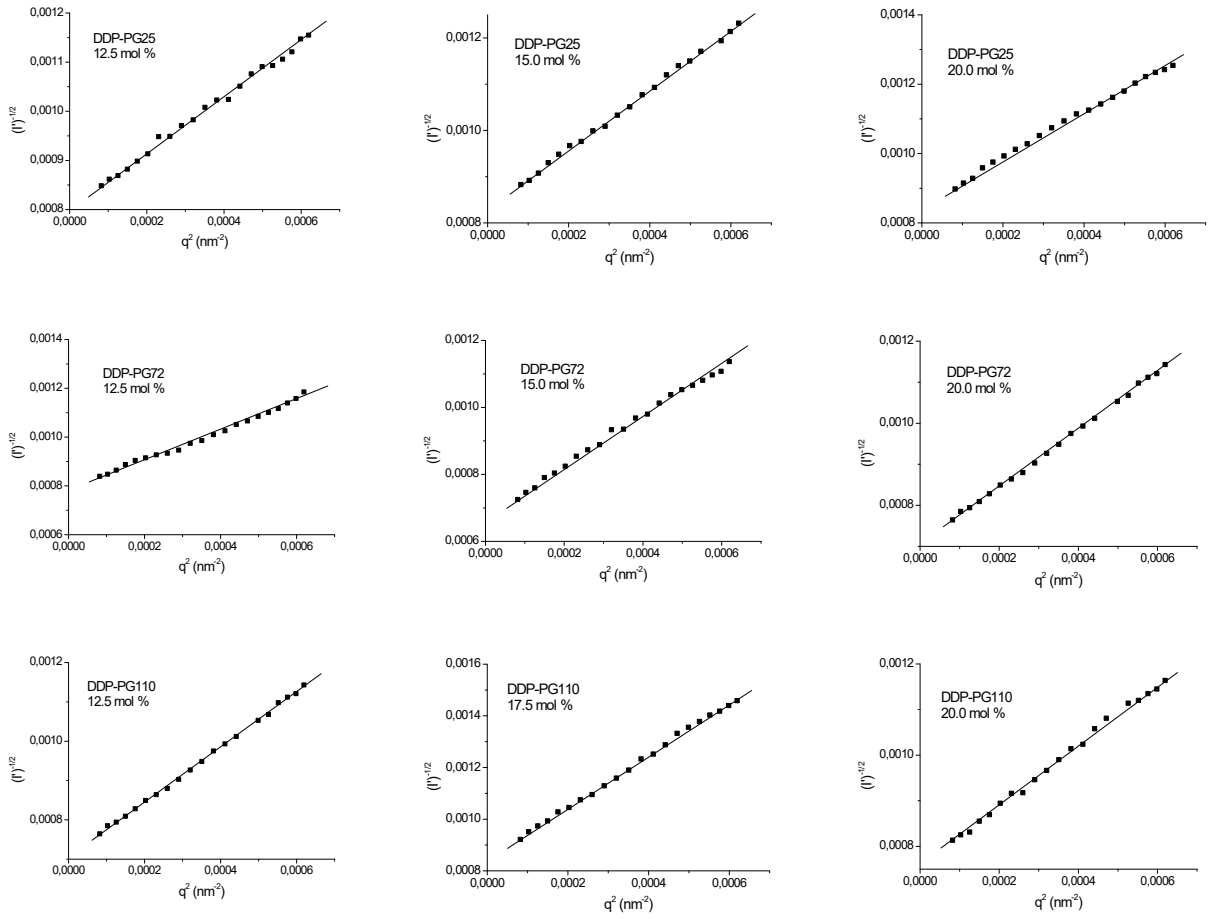


Figure S5. Partial Berry plots for determination of radii of gyration (R_g) of vesicles DPPC/Cholesterol/DDP-PG25 (top row), DPPC/Cholesterol/DDP-PG72 (middle row), and DPPC/Cholesterol/DDP-PG110 (bottom row) at different DDP-PG contents indicated.

- **Electrophoretic Light Scattering**

The electrophoretic light scattering measurements were carried out on a 90Plus PALS instrument (Brookhaven Instruments Corporation) equipped with a 35 mW red diode laser (λ = 640 nm) at a scattering angle (θ) of 15°. ζ potentials were calculated from the obtained electrophoretic mobility at 25 °C by using the Smoluchowski equation (equation 2)

$$\zeta = 4\pi\eta\upsilon/\varepsilon \qquad \text{(equation 2)}$$

where η is the solvent viscosity, υ is the electrophoretic mobility, and ε is the dielectric constant of the solvent.

- **Atomic force microscopy (AFM)**

AFM images were taken on a NanoScope V instrument (Bruker Inc., Billerica, MA) with a 1.00 Hz scan rate under ambient conditions. Topography imaging was performed in tapping mode using silicon nitride (Si_3N_4) probes (Tap300Al-G, Budget Sensors, Sofia, Bulgaria) with tip radius < 10 nm. The images (512×512 pixels) were captured in height, deflection and phase mode and are presented here without any additional processing. For AFM sample preparation, a droplet of 2 µL dispersion was placed onto a freshly cleaned glass substrate (1 cm^2) and spin-casted at 2000 rpm for 1 min.

- **Determination of the viscosity and refractive index of the saline solution**

The values of viscosity and refractive index of the saline solution (0.9 % NaCl) were calculated from the viscosity vs. concentration (Figure S6)[4] and refractive index vs. concentration (Figure S7) data,[5] applying polynomial and linear fits to the data, respectively.

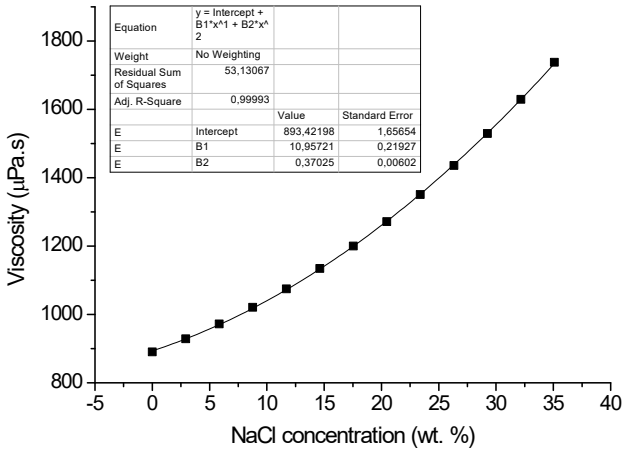


Figure S6. Viscosity vs NaCl concentration at 25 °C. Data taken from ref. 4.

By a polynomial fit of a second order (equation 3), with a = 893.42, b_1 = 10.96, and b_2 = 0.37, at x = 0.9, the value of the viscosity is 903.6 µPa.s (= 0.9036 cP).

$$y = a + b_1x + b_2x^2 \qquad \text{(equation 3)}$$

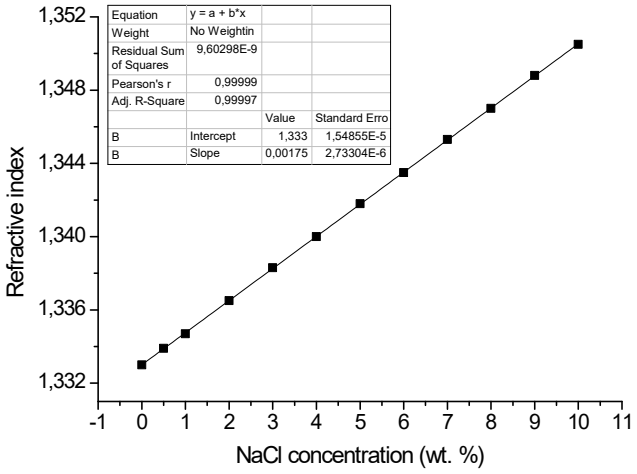


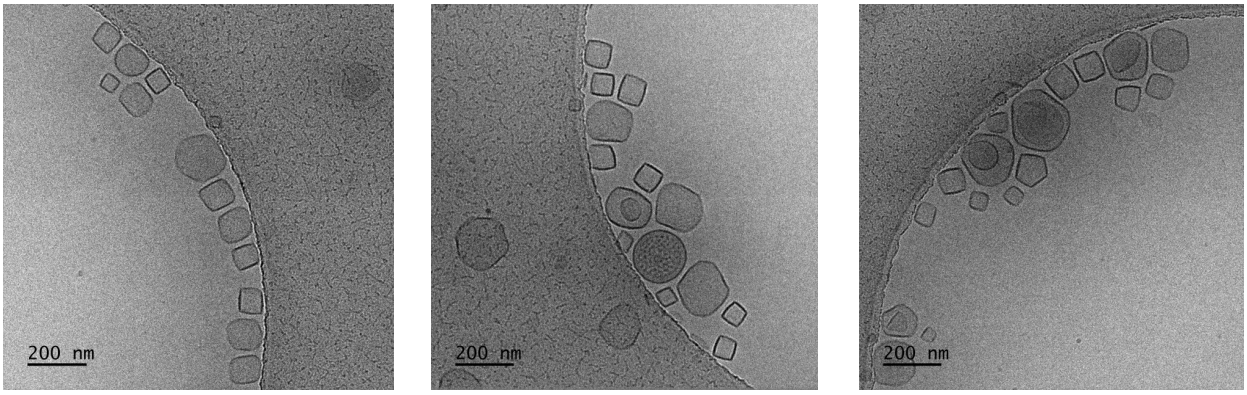
Figure S7. Refractive index vs NaCl concentration. Data taken from ref. 5

By a linear fit (equation 4), with a = 1.333, and b = 0.0018, at x = 0.9, the value of the refractive index is 1.3346.

$$y = a + bx \qquad \text{(equation 4)}$$

- **Gallery of cryo-TEM images of DPPC/Cholesterol vesicular dispersions sterically stabilized by DDP-PG polymers**

Cryo-TEM images of DPPC/Cholesterol vesicular dispersions sterically stabilized by DDP-PG polymers are shown in Figures S8 – S10.



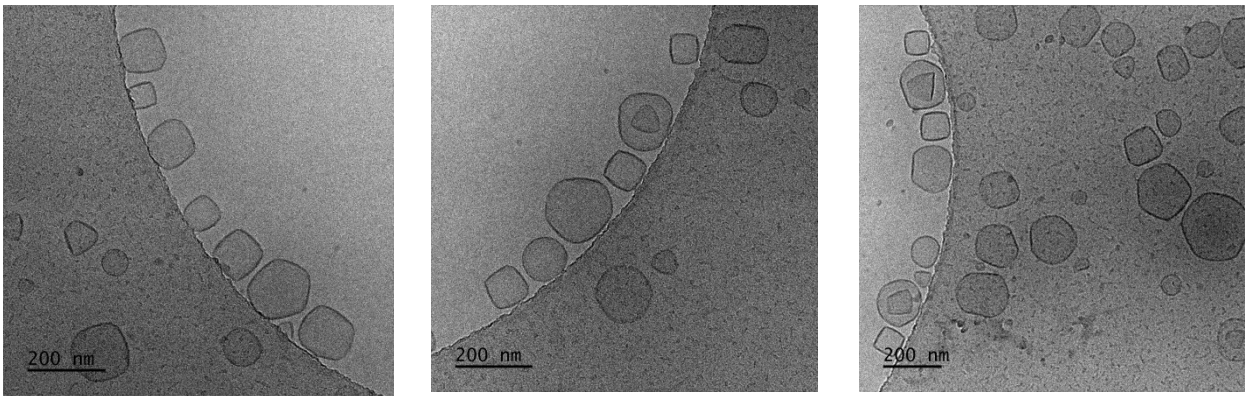


Figure S8. Gallery of cryo-TEM images of DPPC/Cholesterol vesicular dispersions sterically stabilized by 17.5 mol % (upper panel) and 12.5 mol % (lower panel) of DDP-PG110.

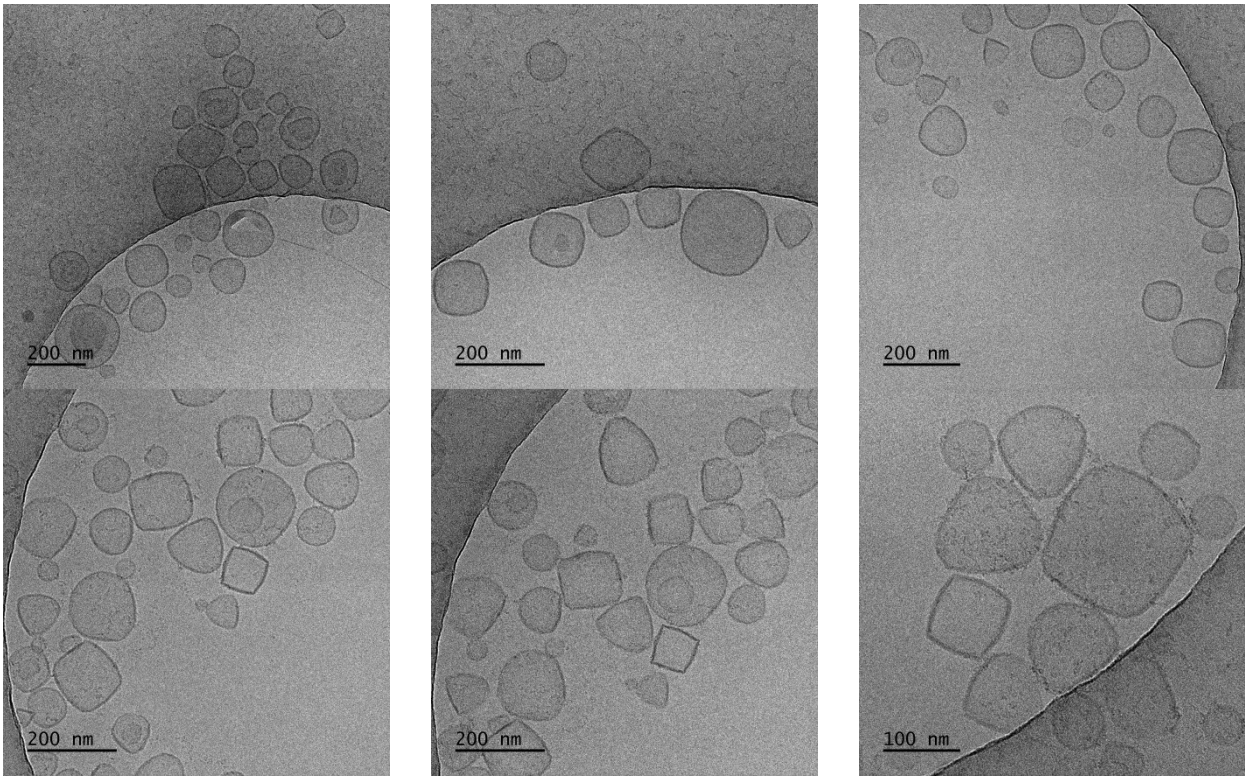


Figure S9. Gallery of cryo-TEM images of DPPC/Cholesterol vesicular dispersions sterically stabilized by 15.0 mol % (upper panel) and 12.5 mol % (lower panel) of DDP-PG72.

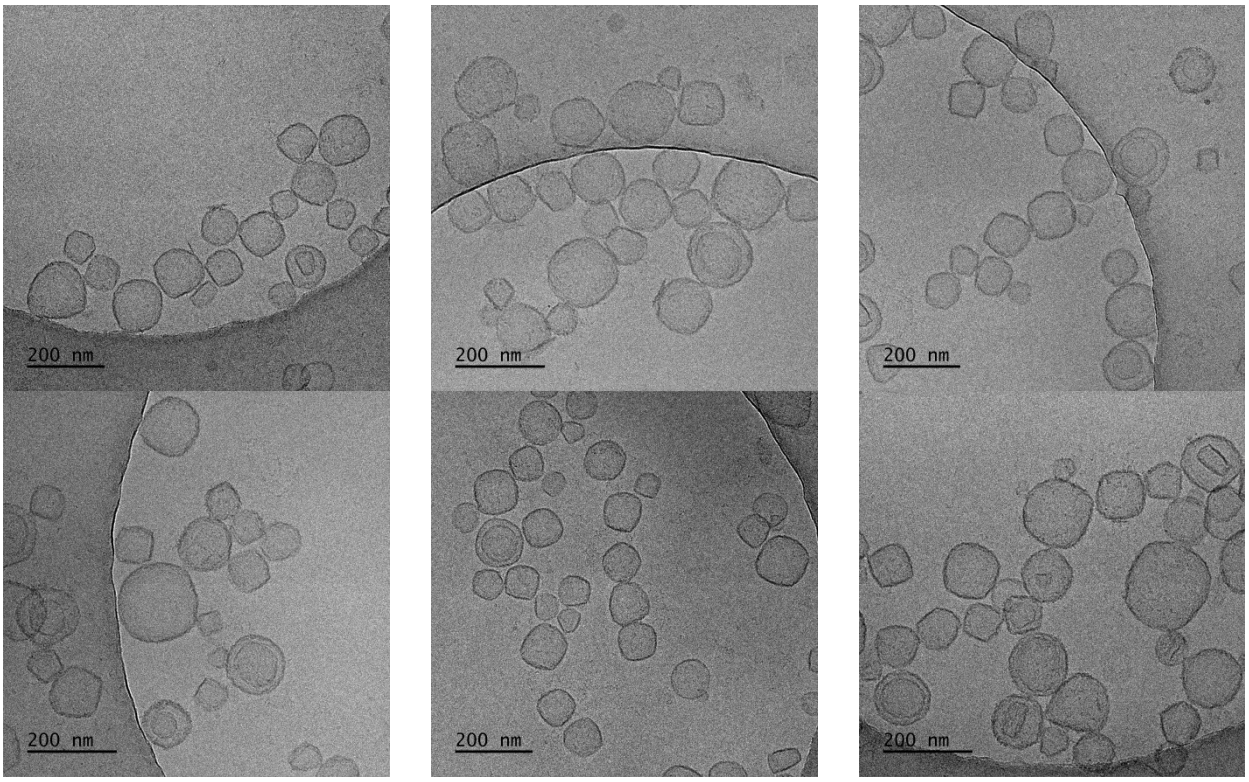


Figure S10. Gallery of cryo-TEM images of DPPC/Cholesterol vesicular dispersions sterically stabilized by 15.0 mol % of DDP-PG25.

- **Calculation of the grafting density of the polyglycidol chains in the bilayer**

The total bilayer area of a vesicle was calculated from $S = 2 \times (4\pi R_h^2)$ and $S = 2 \times (6a^2)$ for the vesicles with dominant spherical and cuboidal morphologies, respectively, where R_h is the hydrodynamic radius from DLS and a is the face length of the vesicle cube from cryo-TEM. Assuming an average area of 0.65 nm^2 per lipid molecule/lipid-mimetic anchor,[6] the aggregation number was calculated, from which the number of polyglycidol chains per vesicle was determined knowing the content of DDP-PG polymers for each formulation. The grafting density of the polyglycidol chains (σ), expressed as a number of polyglycidol chains per nm^2, was calculated from the number of polyglycidol chains and total bilayer area. The results for all liposomal formulations are collected in Table S1.

Table S1. Total bilayer area of a vesicle, aggregation number (N_{agg}), number of polyglycidol chains per vesicle and grafting density of polyglycidol chains of DPPC/Cholesterol liposomes containing different amounts of DDP-PG.

Sample	Dominant Morphology	Size (nm)	Total bilayer area (nm^2)	N_{agg}	Number of polyglycidol chains per vesicle	σ (nm^{-2})
DDP-PG25 – 12.5	Spherical	64.3[a]	103911	159863	19983	0.192
DDP-PG25 – 15.0	Spherical	63.0[a]	99752	153464	23020	0.231
DDP-PG25 – 17.5	Spherical	62.8[a]	99120	152492	26686	0.269
DDP-PG25 – 20.0	Spherical	71.4[a]	128126	197117	39423	0.308
DDP-PG72 – 12.5	Spherical	66.3[a]	110476	169963	21245	0.192
DDP-PG72 – 15.0	Spherical	78.7[a]	155664	239484	35923	0.231
DDP-PG72 – 20.0	Spherical	72.0[a]	130288	200443	40089	0.308
DDP-PG110 – 12.5	Cuboidal	85.0[b]	86700	133385	16673	0.192
DDP-PG110 – 17.5	Cuboidal	85.0[b]	86700	133385	23342	0.269
DDP-PG110 – 20.0	Cuboidal	85.0[b]	86700	133385	26677	0.308

[a] R_h from DLS, [b] an average face length of a cube, determined from a large number of objects from cryo-TEM.

- **Calculation of the Flory radius, R_F, and grafting densities at the mushroom to brush transition for DDP-PG**

The Flory radius is the end-to-end distance of a free polymer coil in a theta solvent, resulting from the balance between the expanding steric forces and the counteracting entropic forces from stretching of the coils. In its simplest form it is given by equation 5:[7]

$$R_F = aN^{3/5} \qquad \text{(equation 5)}$$

Here *a* is the length of the monomer unit and *N* is the degree of polymerization. If the length of the glycidol unit is taken as 0.35 nm, for polyglycidol degrees of polymerization of 25, 72, and 110, one would obtain Flory radius values of 2.4, 4.6, and 5.9 nm, respectively (Table S2).

The polymer coils start to interact laterally when the distance between the grafting points is equal to or less than the Flory radius. Thus, the grafting density at the transition from an unextended (mushroom) to brush conformation can be determined by $\sigma_{tr} = 1/R_F^2$. The values of σ_{tr} are summarized in Table S2. When $\sigma < \sigma_{tr}$ the tethered polymer coils are isolated; if $\sigma > \sigma_{tr}$ they interact laterally, overlap, and adopt a more extended (brush) conformation.

Table S2. Flory radii of the polyglycidol coils and grafting densities at the mushroom to brush transition for DDP-PG.

Polymer	**R_F (nm)**	**σ_{tr} (nm^{-2})**
DDP-PG25	2.4	0.174
DDP-PG72	4.6	0.048
DDP-PG110	5.9	0.029

- **Differential scanning calorimetry (DSC)**

DSC experiments were carried out using DSC Perkin Elmer 8500 with a refrigerated cooling accessory Intercooler. Desired amounts of water at contents near the full hydration[8] were added to sample mass from lyophilized dispersions of DPPC, DDP-PG110, DPPC/cholesterol, DPPC/DDP-PG110, and DPPC/cholesterol/DDP-PG placed in aluminium DSC pans. The pans were hermetically sealed and the DSC study was initiated by program-heating from -5 to 68 °C. A heating rate of 5 °C/min and an inert atmosphere were used. Figure S11 shows DSC thermograms of the nearly fully hydrated samples. The diacyl carbon chain melting was observed for the DPPC-water system at around 42 °C. It completely disappeared upon the addition of cholesterol, DDP-PG110, and cholesterol and DDP-PG110, implying that these systems were in a more fluid, liquid-crystalline phase.

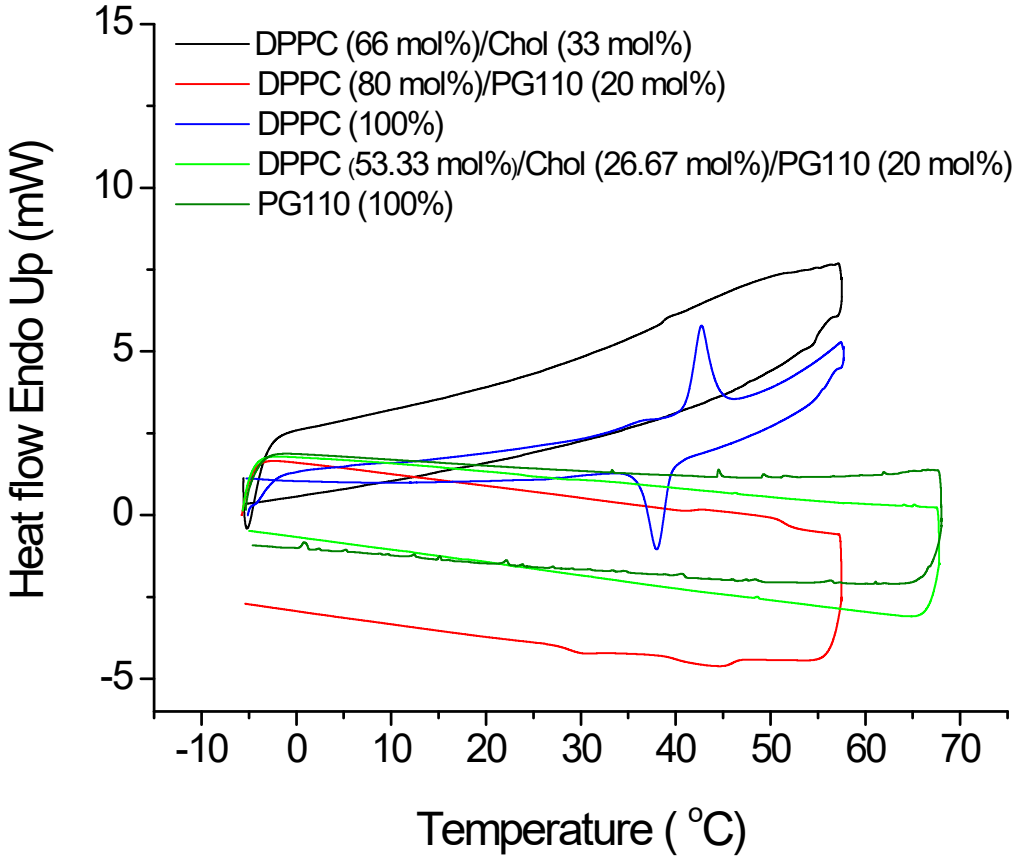


Figure S11. DSC thermograms of the nearly fully hydrated smaples of DPPC, DDP-PG110, DPPC/cholesterol, DPPC/DDP-PG110, and DPPC/cholesterol/DDP-PG

REFERENCES

1. S. Rangelov, E. Petrova, I. Berlinova, Ch. Tsvetanov, *Polymer,* 2001, **42**, 4483-91. https://doi.org/10.1016/S0032-3861(00)00826-0

2. P. Bakardzhiev, S. Rangelov, B. Trzebicka, D. Momekova, G. Lalev, *RSC Advances,* 2014, **4 (70)**, 37208-19. **DOI**: 10.1039/C4RA03102D

3. S. W. Provencher, *Macromol. Chem.,* 1979, **180**, 201–209. https://doi.org/10.1002/macp.1979.021800119

4. J. Kestin, H. E. Khalifa, R. F. Correia, *J. Phys. Chem. Ref. Data,* 1981, **10 (1)**, 71–87. https://doi.org/10.1063/1.555641

5. D. R. Lide, in *Handbook of Chemistry and Physics*, CRC Press, 86th ed. (D. R. Lide ed.), pp. 8 - 71. Boca Raton, FL.

6. J. F. Nagle, S. Tristram-Nagle, *Biochim. Biophys. Acta,* 2000, **1469**, 159–195. DOI 10.1016/s0304-4157(00)00016-2

7. P. J. Flory in *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, N.Y, **1953**.

8. M. Kodama, Y. Kawasaki, H. Aoki, Y. Furukawa, *Biochim. BioPhys. Acta (BBA) – Biomembranes,* 2004, **1667**, 56-66. https://doi.org/10.1016/j.bbamem.2004.08.015